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Transduction of skin-migrating dendritic cells by human adenovirus
5 occurs via an actin-dependent phagocytic pathway.

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18 **ABSTRACT**

19 Dendritic cells (DC) are central to the initiation of immune responses and various
20 approaches have been used to target vaccines to DC in order to improve immunogenicity.
21 Cannulation of lymphatic vessels allows for the collection of DC that migrate from the skin.
22 These migrating DC are involved in antigen uptake and presentation following vaccination.
23 Human replication-deficient adenovirus (AdV) 5 is a promising vaccine vector for delivery
24 of recombinant antigens. Although the mechanism of AdV attachment and penetration has
25 been extensively studied in permissive cell lines, few studies have addressed the
26 interaction of AdV with DC. In this study, we investigated the interaction of bovine skin-
27 migrating DC and replication deficient AdV-based vaccine vectors. We found that despite
28 lack of expression of CAR and other known adenovirus receptors, AdV readily enters skin-
29 draining DC via actin-dependent endocytosis. Virus exit from endosomes was pH-
30 independent and neutralizing antibodies did not prevent virus entry but did prevent virus
31 translocation to the nucleus. We also show that combining adenovirus with adjuvant
32 increases the absolute number of intracellular virus particles per DC but not the number of
33 DC containing intracellular virus. This results in increased trans-gene expression and
34 antigen presentation. We propose that in the absence of CAR and other known receptors,
35 AdV5-based vectors enter skin-migrating DC using actin-dependent endocytosis which
36 occurs in skin-migrating DC and its relevance to vaccination strategies and vaccine vector
37 targeting is discussed.

38

39 INTRODUCTION

40 Vaccines based on replication incompetent adenovirus (AdV) vectors are safe and highly
41 immunogenic, capable of inducing a full spectrum of adaptive humoral and cell-mediated
42 immune responses, and of inducing protective immunity in a number of animal species
43 including man (Dicks *et al.*, 2015; Green *et al.*, 2015; Taylor *et al.*, 2015).

44 Human adenovirus 5 (AdV5 and sometimes referred to as HAdV-C5), a species C
45 adenovirus, is the most commonly studied adenovirus vector for both gene therapy and
46 vaccination and so it is also the most studied in terms of cell entry, host responses and
47 gene expression (Smith *et al.*, 2010). Epithelial cell models have been used to describe
48 the mechanism of AdV5 entry and trafficking to the nuclear membrane (Svensson &
49 Persson, 1984; Wolfrum & Greber, 2013). It is generally accepted that the first step in
50 AdV5 entry to its target cell is the binding of the virus' fiber protein to CAR (Coxsackie B-
51 Adenovirus Receptor), followed by the binding of the RGD motif on the penton base to
52 cellular integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$). This promotes virus endocytosis into clathrin-coated
53 vesicles and triggers the first step of the uncoating program (Burckhardt *et al.*, 2011).
54 Once inside the cell, the virus exits endosomal vesicles to the cytosol, where it utilizes
55 microtubule motors to traffic to the nuclear membrane (Bremner *et al.*, 2009) and deliver
56 its DNA through the nuclear pore (Puntener *et al.*, 2011).

57 Although CAR has been shown to be the primary receptor for AdV5 entry in epithelial cells,
58 CAR is not expressed on all cells that can be infected with AdV5. For example, infection of
59 Kupffer cells is mediated by human blood coagulation factor X binding to AdV5 hexon
60 (Alba *et al.*, 2009; Kalyuzhniy *et al.*, 2008; Waddington *et al.*, 2008) and entry of AdV5 to
61 human peripheral blood monocyte-derived dendritic cells involves CD209 (Adams *et al.*,
62 2009).

63

64 Since their identification (Steinman & Cohn, 1973), dendritic cells (DC) have become
65 increasingly recognized for their crucial role as initiators and regulators of immune
66 responses. Many studies of DC biology rely on the isolation of monocytes or macrophages
67 from blood or tissues (such as spleen or bone marrow) (Rossi & Young, 2005; Steinman,
68 1991), followed by maturation with interleukin 4 (IL-4) and granulocyte-macrophage
69 colony-stimulating factor (GM-CSF), or the harvesting of tissues followed by isolation of
70 resident DC. The cannulation of lymphatic vessels provides *ex vivo* DC derived from
71 relevant anatomical sites such as the skin that drain sites of vaccination (Hemati *et al.*,
72 2009; Hope *et al.*, 2006; Schwartz-Cornil *et al.*, 2006). Due to the complexity of the
73 surgical procedure to cannulate lymphatic vessels, this is most easily performed in large
74 animals, such as cattle and sheep. We and others have described afferent lymph dendritic
75 cells (ALDC) as being FSC^{high} MHCII⁺ DEC-205⁺ CD11c⁺ CD8 α ⁻ (Cubillos-Zapata *et al.*,
76 2011; Gliddon *et al.*, 2004; Hope *et al.*, 2006). Within this population, subpopulations of DC
77 expressing various levels of SIRP α (CD172a), CD11a, CD26 and CD13 have also been
78 described (Brooke *et al.*, 1998; Gliddon *et al.*, 2004; Gliddon & Howard, 2002; Howard *et*
79 *al.*, 1997). Of these, the SIRP α ⁺ DC population is targeted by various vaccine vectors and
80 these cells are more efficient at antigen presentation compared to SIRP α ^{neg/low} ALDC
81 (Guzman *et al.*, 2012; Hope *et al.*, 2012).

82

83 In the current study, we describe the interaction between replication-deficient AdV5 with
84 bovine ALDC that drain the skin. We show that macropinocytosis is the principal entry
85 mechanism for AdV5 into ALDC and that the kinetics of virus internalization are much
86 slower than previously described for epithelial cells. We also show that virus exit from
87 endosomal compartments does not require an acidic microenvironment. Furthermore,
88 neutralizing antibodies do not block internalization of AdV5 but prevent trans-gene
89 expression. Finally, we demonstrate that emulsification of AdV5 in oil-in-water adjuvants

90 improve virus internalization into ALDC *in vitro*, increasing trans-gene expression and
91 antigen presentation. Defining and manipulating entry pathways may enhance vaccine
92 vector efficacy through improved antigen delivery and presentation.

93

94 RESULTS

95 Bovine afferent lymph DC are transduced by adenovirus-based vectors despite the 96 absence of known adenovirus receptors.

97 We have previously shown that AdV5 injected subcutaneously or intramuscularly above
98 the site of cannulation is internalized by migrating DC between 4 and 12 hrs post
99 inoculation and that, *in vitro*, up to 40% of ALDC can be transduced by AdV5 using an moi
100 of 100 (Cubillos-Zapata *et al.*, 2011). To define the mechanism by which AdV5 transduces
101 ALDC (defined as FSC^{high} MHCII⁺ DEC-205⁺ CD11c⁺ CD8α⁻, Fig. 1a), we initially assessed
102 the expression of CAR on bovine cells including ALDC. CAR was detected by Western
103 blotting in enriched membrane fractions from 293 and bovine lung (BL) cells but not
104 membranes from bovine ALDC, (Fig. 1b). We then used the virus overlay binding assay
105 (VOPBA) to confirm binding of AdV5 to bovine CAR. Under denaturing and non-denaturing
106 conditions, AdV5 bound to enriched membrane fractions from 293 and BL cells, but not
107 from ALDC (Fig. 1c and 1d). To confirm that AdV5 was binding specifically to CAR, a
108 rabbit polyclonal antibody raised against CAR was used to block binding of AdV5 to CAR
109 in a competition VOPBA assay (Fig. 1e). These results indicate that although AdV5 can
110 use CAR for binding to BL cells, CAR is not expressed on bovine ALDC and, therefore
111 AdV5 utilises an alternative entry strategy for ALDC that is CAR-independent.

112 A number of different molecules have been implicated in AdV attachment to mononuclear
113 phagocytic cells, including, MHC, CD80/86, CD209 and sialic acid (Chen & Lee, 2014).
114 We used a combination of chymotrypsin, trypsin and papain to remove surface expression
115 of known AdV receptors and brefeldin A to prevent their surface expression during
116 transduction of ALDC. Following this treatment, the viability decreased to between 8 and
117 12% as measured by trypan blue exclusion (data not shown). MHC I (Fig. 2a), MHC II (Fig.
118 2b), CD80 (Fig. 2c), CD86 (Fig. 2d), and CD209, also known as DC-SIGN (Fig. 2e) were

119 completely removed by protease treatment and their reconstitution on the cell's surface
120 prevented throughout the course of the experiments. Removal of sialic acid with
121 neurominidase (Fig. 2f) was also complete. In all cases, these treatments did not result in
122 a decrease transduction efficiency of ALDC by Adv5-GFP (Fig. 2g-l). Blocking the RGD-
123 binding receptors with the antagonist Cyclo(Ala-Arg-Gly-Asp-3-Aminomethylbenzoyl
124 decreased transduction efficiency of 293 cells but not ALDC (Fig. 2m and n). Finally,
125 transduction of both 293 and ALDC was blocked by incubating in the presence of bovine
126 hyperimmune serum against Adv5 (Fig. 2o and p). These results indicate that using the
127 treatments indicated above, transduction of ALDC by Adv5 is not affected.

128 **Transduction kinetics of ALDC.**

129 To investigate transduction kinetics of ALDC by Adv5, we utilised biotinylated Adv5-GFP
130 (moi = 100 vp/cell) to infect freshly isolated ALDC and 293 cells. After a 90 minute
131 incubation at 4°C or 37°C, more than 60% of the biotinylated Adv5-GFP had attached to
132 293 cells (Fig. 3a). In contrast, less than 5% of the biotinylated Adv5-GFP had attached to
133 ALDC at either temperature. We also blocked attachment of Adv5 to 293 cells at both
134 temperatures using anti-CAR antibodies and, as expected, we were able to block 95-98%
135 of the Adv5-bio signal (data not shown). To confirm that biotinylation of the virus did not
136 interfere with its ability to transduce cells and express encoded proteins, we also
137 measured GFP expression in these cells by flow cytometry (Fig. 3b). A 90 minute
138 incubation of 293 cells with biotinylated Adv5 at +4°C or 37°C, followed by washing off the
139 excess inoculum and subsequent overnight incubation, was sufficient to transduce more
140 than 80% of the cells. In contrast, <1% of ALDC expressed GFP following the same
141 protocol. To determine the minimum time required for transduction of cells by Adv5, 293
142 cells and ALDC were incubated with Adv5-GFP at 37°C, the inoculum was washed off at
143 one hour intervals, and the cells were incubated overnight. Transduction was measured by
144 assessing the percentage of cells expressing GFP by flow cytometry. We determined that

145 1 hr incubation was sufficient for AdV5 to transduce and express GFP in 293 cells but that
146 at least 5 hrs were required for ALDC to be significantly transduced by the virus (Fig. 3c).
147 To determine the kinetics of transgene expression, ALDC cells were cultured with AdV5-
148 GFP (moi = 100) at 37°C without washing, and GFP expression was measured at various
149 time points by flow cytometry (Fig. 3d). GFP-positive 293 cells were evident at 4 hrs post
150 transduction and by 8 hrs most cells were expressing the transgene. In contrast, at least a
151 12 hr incubation period was required before a significant number of ALDC expressed GFP
152 (Fig. 3d, $p = 0.0071$ %GFP expression in ALDC at 12 hrs compared to GFP expression at
153 time 0). Expression of GFP in ALDC peaked after 24 hrs in culture. Interestingly,
154 transgene expression in ALDC subsequently decreased and approached baseline levels
155 by 48 hrs (Fig. 3d). These results indicate that the mechanism of AdV5 entry and trans-
156 gene expression in ALDC is significantly different to that in CAR-expressing 293 cells.

157 **ALDC actively uptake adenovirus.**

158 We then analysed the ability of a number of endocytosis inhibitors to block AdV5 entry and
159 subsequent transduction. A fluorometric assay based on the capacity of trypan blue to
160 quench extracellular but not intracellular fluorescein (Wan *et al.*, 1993) was modified to
161 determine the effect of various biochemical inhibitors on the capacity for ALDC to
162 internalize fluorescein-labelled AdV5 (AdV5-Fluo). Incubation at 4°C for 60 min followed by
163 quenching with trypan blue blocked internalization of AdV5-Fluo and no fluorescence was
164 detected in either cell type (Fig. 4b). All other treatments were carried out as described in
165 Materials and Methods. In comparison with DMSO, treatment with the various inhibitors
166 resulted in the following: treatment with filipin, which blocks caveolae and cholesterol-
167 dependent endocytosis, and chlorpromazine (CPZ), which inhibits clathrin-dependent
168 endocytosis, reduced AdV5-Fluo uptake by 293 cells, as expected, but not by ALDC. In
169 contrast, treatment with cytochalasin D (CCD), which blocks actin polymerization and
170 amiloride, which blocks Na^+ channels, both reduced the uptake of virus by ALDC (Fig. 4a

171 and b). Treatment with methyl- β -cyclodextrin (m β -CD), which blocks cholesterol-
172 dependent phagocytosis, did not have an effect on virus uptake in either cell type.
173 Treatment with endocytosis inhibitors at the concentrations observed did not significantly
174 increase the number of dead cells during the course of the assay (data not shown). This
175 data indicates that actin polymerization and Na⁺/H⁺ exchange are required for AdV5
176 uptake by ALDC.

177 **Transient association of AdV5 with early endosome markers.**

178 Following clathrin-mediated endocytosis of AdV5 in 293 cells, virus can be detected within
179 clathrin-coated vesicles (Ashbourne Excoffon *et al.*, 2003). These early endosomes are
180 characterized by the presence of Rab5 and EEA1 (Christoforidis *et al.*, 1999). To
181 determine if entry of AdV5 into ALDC was associated with early endosomes, purified
182 ALDC were cultured with AF568-labelled AdV5 for 1 to 6 hrs and analysed by confocal
183 microscopy. AdV5 could not be detected on the surface of ALDC until 3 hrs after addition
184 of virus when it was found to be associated with dendrites or cell membranes (Fig. 5a). An
185 hour later a greater number of virions were associated with the dendrites/cell membrane
186 and virions were also observed within the cytoplasm (Fig. 5b). By 5 hrs post infection, a
187 proportion of virions were localized in proximity to the nucleus (Fig. 5c). We then stained
188 AdV5-infected ALDC with EEA1-specific antibodies to determine localization of AdV5 with
189 early endosomes. Although co-localization of AdV5-AF568 with EEA1 was observed at 4
190 hrs post infection (Fig. 6a), co-localization events were rare, suggesting that virus exit from
191 the phagosome/endosome occurs quickly after entry or that the majority of AdV are not
192 associated with early endosomes. We then tracked the localization of AdV5-AF568 within
193 ALDC using two different AF488-labelled tracers: dextran is used to track fluid-phase
194 pinocytosis and albumin is known to enter cells using a mannose receptor-endocytosis
195 pathway. We observed co-localization of AdV568 with dextran-AF488 but not with

196 albumin-AF488 (Fig. 6c-d), suggesting that AdV5 entry into ALDC is via fluid-phase
197 macropinocytosis.

198 **Exit of AdV5 into the cytosol of ALDC is not pH-mediated.**

199 Following uptake, a key step in AdV5 infection is the exit of virus or virus aggregates from
200 phagosomes/endosomes into the cytoplasm (Meier *et al.*, 2005). To define the
201 mechanisms associated with AdV5 exit in ALDC, cells were treated with inhibitors of the
202 intracellular acidic microenvironment such as bafilomycin, NH₄Cl or chloroquine, 60 min
203 prior to the addition of AdV5-GFP. Cells were then cultured for 4 hrs and GFP expression
204 measured by flow cytometry. None of the treatments significantly reduced the proportion of
205 ALDC or 293 cells expressing GFP (Fig. 7A). To confirm the presence of acidic
206 endosomes within ALDC, we utilized fluorescein-labelled dextran and fluorescein-labelled
207 AdV5. The fluorescence of fluorescein is optimal at pH7.5 and rapidly decreases in acidic
208 conditions. Under normal culture conditions, the fluorescence intensity (MFI) of
209 fluorescein-labelled dextran within ALDC decreases after a 4 hr incubation (Fig. 7b),
210 confirming the presence of dextran within endosomes and their subsequent acidification.
211 In contrast, fluorescein-labelled AdV5 within ALDC did not show a decrease in
212 fluorescence after a 4 hr incubation (Fig. 7b). As expected, normalization of intracellular
213 pH with 10 mM NH₄Cl, pH7.5, inhibited the reduction of fluorescence in dextran-loaded
214 ALDC. To confirm these results, we measured the relative fluorescence of fluorescein at
215 various pH and generated a standard curve (Fig. 7c). ALDC were incubated with
216 fluorescein-labelled AdV5 or dextran and fluorescence was measured by real time
217 fluorometry. Figure 7d shows that the fluorescence of dextran in ALDC decreases over
218 time as the fluorescein becomes protonated, but the fluorescence of AdV5 remains the
219 same suggesting that acidic endosomes are not involved in adenovirus uncoating in
220 ALDC.

221 **Neutralizing antibodies do not prevent virus entry into ALDC.**

222 An important hurdle to successful vaccination with viral vectors is the presence of
223 neutralizing antibodies which can limit the efficacy of the vaccine. Most neutralizing
224 antibodies are raised against the fibre protein of AdV and thus block virus attachment to its
225 receptor (normally CAR). In light of our results, we sought to investigate the effect of
226 neutralizing antibodies on AdV5 entry into ALDC. We incubated AdV5-568 with normal
227 bovine sera, hyperimmune bovine sera to AdV5 or mouse monoclonal anti-hexon
228 antibodies. Virus-Ab complexes were then added to cultures of ALDC and virus entry
229 assessed by confocal microscopy, 4-6 hrs post infection. Interestingly, the mean number
230 of intracellular AdV5 was significantly higher ($p=0.0027$) in the presence of bovine
231 hyperimmune sera or mouse anti-hexon antibodies compared to normal bovine sera (S1a).
232 However, even after 6 hrs in culture, virus complexed with antibody did not migrate to the
233 nuclear membrane, but remained in the mid-cytoplasm (S1b) whereas AdV5, incubated
234 with normal bovine sera, migrated and was located proximal to the nuclear membrane as
235 expected (S1c). This data shows that the presence of neutralising antibodies does not
236 prevent attachment of the virus to the cell but rather block a process downstream of virus
237 penetration.

238 **Oil-in-water adjuvants increase virus uptake and enhance antigen expression.**

239 Various approaches have been proposed to improve targeting of AdV vectors to DC,
240 including modification of the virus fibre protein to increase virus binding and penetration to
241 DC. Previous observations by us and others (Cubillos-Zapata *et al.*, 2011; Ganne *et al.*,
242 1994) indicate that the use of oil-in-water emulsions as adjuvants to deliver AdV5-based
243 vaccines significantly increases the magnitude of immune responses to the trans-gene *in*
244 *vivo*. To identify the effect of adjuvanted vector on ALDC, AF568-labelled AdV5-GFP was
245 prepared in an oil-in-water emulsion, or combined with the adjuvant without mixing, and
246 incubated ALDC with the preparations. ALDC incubated with emulsified virus contained a

247 significantly greater number of intracellular virions compared to cells incubated with virus
248 in adjuvant without mixing or in PBS ($p = 0.0248$, Figures 8a, b and c). The mean
249 fluorescence intensity of GFP was greater (Fig. 8d) and expression was more sustained
250 (Fig. 8e) in ALDC incubated with emulsified virus than in cells incubated with the virus in
251 adjuvant without mixing or in PBS. However, the percentage of cells expressing the trans-
252 gene did not change (Fig. 8e). To confirm the effect of adjuvanted vector on antigen
253 presentation, AdV5 expressing mycobacterial antigen 85A (Ag85A, (Cubillos-Zapata *et al.*,
254 2011)) in adjuvant was prepared with or without mixing and incubated with ALDC. These
255 ALDC were then cultured with CD4⁺ T cells obtained from MHC-matched, BCG-vaccinated
256 cattle. Ag85A-specific responses were significantly higher when AdV5-Ag85A was mixed
257 with adjuvant compared to AdV5-Ag85A without mixing or without adjuvant ($p = 0.0076$).
258 The number of IFN- γ producing cells was minimal when T cells were cultured with ALDC
259 exposed to AdV5-GFP with or without adjuvant, or to adjuvant alone (Fig. 8f). This data
260 shows that in the absence of genetic modification of the virus fibre, increased transduction
261 efficiencies can be achieved by the use of water-in-oil adjuvants.

262 DISCUSSION

263 We have previously shown that in contrast to monocyte-derived DC, ALDC can be readily
264 transduced by replication-deficient AdV5 (Cubillos-Zapata *et al.*, 2011), achieving up to
265 50% transduction of ALDC *in vitro* and up to 12% *in vivo*. ALDC can be separated into two
266 main subpopulations, CD172 α ⁺ and CD172 α ⁻; only the former can be transduced by AdV5
267 vectors both *in vitro* and *in vivo* (Cubillos-Zapata *et al.*, 2011). In the current study, we
268 aimed to characterize the mechanism of AdV5 uptake by ALDC further.

269 Since its discovery as a primary receptor for AdV5 (Bergelson *et al.*, 1997), it has been
270 widely accepted that CAR is involved in AdV5 attachment to target cells. However, a
271 number of other cell surface molecules, such as CD40, MHC II, CD46 and Fc receptors,

272 have been identified which appear to play an important role in AdV5 attachment (reviewed
273 in (Zhang & Bergelson, 2005)). We could not prevent the transduction of ALDC by AdV5
274 following proteolytic cleavage of surface proteins or by using RGD-blocking peptides.
275 Although there is evidence that DC-SIGN is involved in attachment of AdV5 to human
276 monocyte-derived DC (Adams *et al.*, 2009), we could not replicate these results using
277 bovine ALDC. This may be due to differences between the interaction of AdV5 between
278 human and bovine DC or to differences between monocyte-derived DC and ALDC. Apart
279 from an antibody against CD64 (FcγR), antibodies against bovine FcγR are not available,
280 so it is possible for these receptors to be involved in AdV5 entry into bovine ALDC.
281 VOPBA has been used to identify the binding of dengue virus (Jindadamrongwech &
282 Smith, 2004), pseudorabies virus (Karger & Mettenleiter, 1996) and respiratory syncytial
283 virus (RSV) (Tayyari *et al.*, 2011) to cell receptors. We used this assay under denaturing
284 and non-denaturing conditions to determine if a protein receptor for AdV5 on membrane-
285 enriched fractions from ALDC could be identified. Although binding of virus to a protein
286 present in enriched membrane fractions from 293 and BL cells could be detected, we
287 could not identify binding of AdV5 to membrane proteins derived from ALDC (Figure 1). It
288 is possible that our inability of identify an AdV5 receptor on ALDC is due to the stringency
289 of the assay's conditions or the sensitivity of our assays. New technologies developed to
290 identify protein-protein interaction, such as FRET or high affinity co-immunoprecipitation
291 followed by highly sensitive mass spectrometry may help identify receptors involved in
292 AdV attachment in the future.

293 Using biotinylated AdV5 we showed that the virus does not bind to the surface of ALDC as
294 it does to the surface of 293 cells suggesting that internalization of AdV5 by bovine ALDC
295 is mediated by CAR-independent macropinocytosis and similar pathways have been
296 described for internalization other viruses such as influenza (de Vries *et al.*, 2011) and
297 vaccinia (Sandgren *et al.*, 2010) by DC. Alternatively, it is possible for AdV5 to use low-

298 affinity receptors present on the surface of ALDC as observed in other systems, such as
299 CD46 (Sirena *et al.*, 2004), α M β 2 and α L β 2 (Huang *et al.*, 1996) integrins. It is possible
300 that labelling the virus with sulfo-NHS conjugates (biotin, AF569, fluorescein) changes the
301 way the virus enters the cells. We have tried to address this possibility by using non-
302 labelled virus as control and GFP as readout of transduction when at all possible.

303 Professional phagocytic cells such as DC have the capacity to take up small and large
304 particles using a variety of mechanisms such as macro- and micropinocytosis (Platt *et al.*,
305 2010; Savina & Amigorena, 2007). Using biochemical inhibitors of endocytosis, we found
306 that cytochalasin D (CCD) was able to block AdV5 uptake indicating that AdV5
307 internalization by ALDC is an actin-mediated process (Cooper, 1987), and that skin-
308 migrating DC utilize their capacity as professional phagocytic cells to survey peripheral
309 sites acquiring foreign antigens such as vaccine vectors, and processing these antigens
310 prior to and on arrival to local draining lymph nodes. Interestingly, dynasore had no effect
311 on endocytosis of AdV5 indicating that this process is clathrin-independent (Chen *et al.*,
312 2009) and therefore does not require the binding of RGD to cellular integrins. This
313 suggestion is supported by the finding that RGD blocking peptides did not inhibit AdV5
314 internalization by bovine ALDC (Figure 2).

315 We then looked at events following virus internalization, and using confocal microscopy we
316 observed occasional co-localization of AdV5 particles with the early endosome marker
317 EEA1, but not with the late endosome marker LAMP1 (data not shown), supporting
318 previous evidence of AdV5 exit from early endosomes to the cytosol (Svensson &
319 Persson, 1984). Although it has been previously proposed that AdV5 exits early
320 endosomal compartments following intra-endosome acidification (Greber *et al.*, 1993) and
321 reviewed in (Smith *et al.*, 2010), we could not block virus transduction of ALDC using a
322 number of lysosomotropic agents. Additionally, the fluorescence intensity of AdV5-
323 fluorescein remained constant in ALDC over time whereas the fluorescence intensity of

324 fluorescein-labelled dextran declined as fluorescein became protonated (Figure 7b and d).
325 Our data indicates that acidification of endosomes is not required for transduction of ALDC
326 by AdV5 and this has been shown to be the case in other systems (Otero & Carrasco,
327 1987; Rodriguez & Everitt, 1996; Svensson & Persson, 1984), and although Suomalainen
328 *et al.* found in epithelial cells that virus penetration is independent of low endosomal pH, it
329 could still be inhibited by ammonium chloride; so it is possible that AdV5 exit to the cytosol
330 requires acidification of endosomes in some cells but not others. This raises the question
331 of how AdV5 exits endosomal compartments in ALDC. It has been proposed that viral
332 (Suomalainen *et al.*, 2013) or cellular proteases degrade early endosomes (Wiethoff *et al.*,
333 2005) or perhaps a yet unknown mechanism is involved in this process and requires
334 further investigation.

335 The presence of naturally-acquired neutralizing antibodies against AdV5 is one of the
336 major obstacles in the deployment of effective recombinant AdV5 vaccine vectors (Ahi *et*
337 *al.*, 2011). Antibodies bound to neutralizing epitopes on the virus surface normally prevent
338 virus binding to the cell's receptors (Roy *et al.*, 2005; Sumida *et al.*, 2005). However, we
339 observed that AdV hyperimmune bovine sera enhanced the uptake of AdV5 by ALDC,
340 perhaps through the use of Fc receptors while blocking trans-gene expression. We could
341 not test the effect of blocking Fc receptors due to the lack of available reagents to use in
342 bovine cells. In the presence of AdV5-specific antibody, the AF568-labelled virions were
343 not translocated to the nuclear membrane, but remained in the cytoplasm and the signal
344 was eventually lost (data not shown). A similar phenomenon has been described
345 previously in HeLa cells (Smith *et al.*, 2008), in which TRIM21 binds to antibody-AdV5
346 complexes and targets the complexes for proteasomal degradation (Mallery *et al.*, 2010).
347 The role of TRIM21 in AdV5 degradation remains to be investigated in DC.
348 Various approaches have been proposed to improve targeting of AdV vectors to DC,
349 including modification of the virus fibre protein to increase virus binding and penetration to

350 DC. In light of our current results and previous studies that have shown that AdV5
351 emulsified in an oil-in-water adjuvant and injected over the site of cannulation provides
352 longer trans-gene expression and improved immunogenicity compared to non-adjuvanted
353 virus (Cubillos-Zapata *et al.*, 2011), we sought to understand the mechanism of improved
354 immunogenicity in the absence of clear virus fibre-receptor interactions. Cells transduced
355 with adjuvanted virus contained more intracellular virions and trans-gene expression was
356 stronger and longer-lasting than in cells transduced with virus in the presence of adjuvant
357 but without emulsification, or in PBS. In presentation assays, antigen-specific IFN- γ T cell
358 responses were higher when adjuvanted virus was used. This suggests that the oil-in-
359 water emulsion provides a biological medium which is used by the DC to take up larger
360 amounts of solute, in this case AdV5. This in turn translates into greater numbers of
361 intracellular virions that have the capacity to translocate to the nucleus more effectively
362 and for longer periods of time, or that the virions may be protected from intracellular
363 degradation for a longer period of time when taken up in an adjuvant emulsion. This
364 results in stronger trans-gene expression and thus antigen presentation. In addition, the
365 adjuvant emulsion may activate TLRs which provide signals for the DC to be more
366 effective at activating T cells. However, our controls suggest that this may not be the case
367 since *in vitro* responses to AdV5-Ag85 in PBS are not significantly higher than responses
368 to AdV5-Ag85 in adjuvant but without mixing (Figure 8f). Further studies are required to
369 understand the relationship between biochemical adjuvants and DC. Ultimately, genetic
370 modification of fibre protein will only be useful if a clear cellular receptor is identified in the
371 target cell, therefore alternative approaches, such as oil-in-water emulsions, may be the
372 most appropriate to improve AdV-based gene delivery.

373

374 In conclusion, here we describe the interaction of a replication-deficient AdV vector with
375 skin-migrating bovine DC, which are collected by cannulation of lymphatic vessels and are

not subject to culture in laboratory conditions. We present evidence of the phagocytic action of these DC. Upon encountering virus, ALDC actively phagocytose the virus particles, perhaps using an unknown low-affinity receptor, and which takes between 3 and 4 hrs before virus particles can be observed intracellularly. Following entry, the virus quickly exits endosomal compartments via an unknown mechanism or is never associated with acidic endosomes, travelling to the nuclear membrane and so initiating trans-gene transcription and translation. Neutralizing antibodies do not only prevent virus entry into DC but enhance it whilst inhibiting translocation to the nucleus. Our data will be useful in understanding DC-vaccine interactions and will help further development and improvement of viral vectors. Defining and manipulating entry pathways may enhance vaccine vector efficacy through improved antigen presentation.

METHODS

Pseudoafferent lymphatic cannulation

MHC-defined (Ellis *et al.*, 1996; Ellis *et al.*, 1998), conventionally reared, 6 month-old Friesian Holstein calves (*Bos taurus*) from The Pirbright Institute (Pirbright) herd were used for these studies. Cannulations were performed essentially as described previously (Hope *et al.*, 2006). Lymph was collected into sterile plastic bottles containing heparin (10 U/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml). The lymph collected was either used fresh or was centrifuged (300 x g, 8 min), resuspended in heat-inactivated foetal calf serum (FCS, Autogen Bioclear) containing 10% DMSO and the cells stored in liquid nitrogen prior to use. Mononuclear cells were isolated from afferent lymph by density gradient centrifugation over Histopaque 1083 (Sigma). *Mycobacterium bovis* Ag85A-specific T cells were obtained from MHC-defined cattle vaccinated subcutaneously with 10⁶ CFU of BCG Pasteur. All T cells used were collected 3 weeks post vaccination at the

400 peak of the response. All animal experiments were approved by the Pirbright's ethics
401 committee and carried out according to the UK Animal (Scientific Procedures) Act 1986.

402 Cell lines and primary cells.

403 293 and HeLa cells were obtained and maintained by the Microbiological Services
404 Department (Pirbright) in tissue culture media (TCM) in the absence of antibiotics. CHO
405 cells expressing human recombinant CAR (CHO-CAR) were provided by Dr M.
406 Cottingham, Jenner Institute, University of Oxford, UK. Bovine ALDC (FSC^{high} MHCII⁺
407 DEC-205⁺ CD11c⁺ CD8 α ⁻) were separated from other lymph-migrating cells using a
408 FACSARIA II (Becton Dickinson) and purities were confirmed by flow cytometry using
409 FACSDiva v6 (Becton Dickinson). Peripheral blood CD14⁺ monocytes, CD4⁺ and CD8⁺ T
410 cells were magnetically separated using anti-human CD14 (Miltenyi Biotech), CC30 and
411 CC63 mAbs (Guzman *et al.*, 2008) respectively and MACS technology (Miltenyi Biotech,
412 Germany) following the manufacturer's instructions. Typically, the purity of the resulting
413 dendritic and T-cell subsets was over 97% as determined by flow cytometry as described
414 above.

415 Bovine lung (BL) cells were isolated from Holstein cattle at the time of slaughter and were
416 provided by Pirbright's Microbiological Services Department (Chanter *et al.*, 1986).

417 Monoclonal antibodies and flow cytometry

418 Fluorochrome-labelled mouse anti-bovine monoclonal antibodies (mAb) used in this study
419 have been described in detail previously (Brooke *et al.*, 1998; Howard *et al.*, 1991; Howard
420 & Naessens, 1993; Howard *et al.*, 1997; Sallusto *et al.*, 1995). These were CC98-APC
421 (anti-DEC-205), CC14-PE (anti-CD1b), CC149-PerCP/Cy5.5 (anti-SIRP α), IL-A16-
422 AlexaFluor 680/PE (anti-CD11c), IL-A21-PE (anti-MHCII), ILA-88-FITC (anti-MHC I), IL-
423 A156-PE (anti-CD40), N32/52-3 –PE (anti-CD80) IL-A159-PE (anti-CD86), CC30-

424 APC/Cy5.5 (anti-CD4), CC63-APC/Cy7 (anti-CD8), IL-A111-AlexaFluor 610/PE (anti-
425 CD25), CC302-PE (anti-IFN- γ). Isotype- and concentration-matched anti-turkey
426 rhinotracheitis virus mAbs were used as controls (Hope *et al.*, 2006; Whelan *et al.*, 2003).
427 Dead cells were excluded using the 405 nm excitable dye Live/Dead Aqua or propidium
428 iodide (Invitrogen) following the manufacturer's instructions. The cells were acquired using
429 an LSRFortessa (Becton Dickinson) and staining was analysed using FCS Express v4
430 (DeNovo Software). Afferent lymph DC were distinguished from other cells on the basis of
431 their high Forward Scatter (FSC), expression of MHC II, CD11c and high intensity
432 expression of DEC-205 and lack of CD8 α (Gliddon *et al.*, 2004; Hope *et al.*, 2006). Only
433 live, single events were used for analysis.

434 Viruses

435 E1 and E3-deleted recombinant human AdV5 expressing green fluorescent protein (GFP)
436 or mycobacterial antigen 85A (Ag85A) were generated by the Jenner Institute Viral Vector
437 Core Facility, University of Oxford, UK, as described previously (Cubillos-Zapata *et al.*,
438 2011).

439 For some assays, aliquots of 1×10^{11} vp AdV5 were labelled with NHS-AlexaFluor568,
440 NHS-biotin or NHS-fluorescein (Invitrogen) following the manufacturer's instructions and
441 labelled virus was dialyzed twice against PBS. The virus was then titrated in 293 cells and
442 GFP expression measured by flow cytometry. In some cases the titer was found to be 1
443 log lower after labelling and the infectious doses were adjusted as required.

444 Generation of bovine hyperimmune sera to AdV5

445 Three six month old Holstein-Friesian calves were inoculated intramuscularly with 1×10^9
446 vp of purified AdV5 three times at six week intervals. The hyperimmune sera used was

447 collected 6 weeks after the last immunization, pooled, and tested in a virus neutralization
448 assay (Sumida *et al.*, 2005).

449 Infection of afferent lymph cells

450 Migrating cells from the afferent lymph were cultured (IMDM containing 10% FCS and 10^{-5}
451 M 2- β -mercaptoethanol (Sigma-Aldrich, Poole, UK) with the recombinant viruses using
452 optimal multiplicities of infection, as described previously (Cubillos-Zapata *et al.*, 2011). In
453 some assays, transferrin-AF568 (Sigma) and dextran-fluorescein (Invitrogen) were used
454 as tracing markers. In neutralization assays, AdV5 was incubated with 1 μ g of anti-hexon
455 antibody raised in goats (Millipore) or with bovine pre-immune or hyperimmune sera raised
456 against human AdV5 generated by immunizing calves with 1×10^9 vp of AdV5 as described
457 above.

458 Detection of known adenovirus receptors and removal from cell surface

459 To analyse the expression of known adenovirus receptors on ALDC and bovine lung cells
460 the following antibodies were used in flow cytometry: N-17 (goat anti-CAR, Santa Cruz
461 Biotechnology), N32/52-3 (anti-CD80), ILA-159 (anti-CD86), IL-A88 (anti-MHC class I), IL-
462 A21 (anti-MHC class II), T320.11 (anti-heparin/heparin sulphate, Merck), 2-2B (anti-sialic
463 acid, Merck), CC30 (anti-CD4), CC63 (anti-CD8), 344519 (anti-CD46, R&D Systems),
464 CCG24 (anti-Fc γ RII), KD1 (anti-Fc γ RIII, Abcam), C-20 (anti-DC-SIGN, Santa Cruz
465 Biotechnology), LM609 (anti- $\alpha_v\beta_3$, Chemicon), mAb 2000 (anti- $\alpha_v\beta_1$, Chemicon), 10D5
466 (anti- $\alpha_v\beta_6$, Millipore). All antibodies were obtained from Pirbright except where noted.
467 Antibodies were added to cells (1μ g/ 10^6 cells) and incubated at 4°C for 60 min. After 3
468 washes with PBS the cells were stained with AF647-labelled goat, rabbit or mouse-specific
469 secondary antibodies (Serotec) and the cells analysed by flow cytometry as described
470 above.

471 Digestion of cell surface proteins was achieved using a mixture of proteolytic enzymes
472 (Wald *et al.*, 2001) consisting of 2U of trypsin, 1U of papain, 2U of chymotrypsin (Merck).
473 10^6 cells were treated with the enzyme mix in a volume of 100 μ l for 30 minutes at 37°C;
474 the cells were then washed twice in cold PBS and resuspended in culture media
475 containing a final concentration of 5 μ g/ml of brefeldin A (Sigma).

476 Virus attachment assay by ELISA

477 BL cells, ALDC, CD14⁺ monocytes or 293 cells were cultured on 96-well plates. Antibodies
478 against known AdV5 receptors were added to the cells at 1 μ g/ 10^6 cells; in some cases the
479 following chemical agents known to block AdV5 entry were also added: 10 U of sodium
480 heparin (Sigma), 1 U of trypsin (Sigma), or 10 mM RGD antagonist Cyclo(Ala-Arg-Gly-
481 Asp-3-Aminomethylbenzoyl (Sigma). After 1 hr incubation at 37°C the cells were washed
482 in cold PBS, biotinylated AdV5 (moi=100 vp/cell) was added for 90 minutes on ice or at
483 37°C. The cells were then washed three times with ice-cold PBS and fixed with 3%
484 paraformaldehyde (PF). After blocking with 1% bovine serum albumin (BSA) in PBS,
485 streptavidin-HRP (Sigma, 1:500) was added and the plates incubated for 60 min at room
486 temperature. The plates were washed with PBS-Tween and the plates developed with
487 TMB Turbo (Pierce). Reactions were stopped with 1 M H₂SO₄ and optical densities were
488 measured using a FluorostarOptima (BMG Labtech, Germany).

489 Virus overlay protein binding assay (VOPBA) and Western blot.

490 VOPBA was carried out essentially as described by (Cao *et al.*, 1998) with a few
491 modifications. Subcellular fractions from 1×10^6 293, BL and ALDC were enriched using the
492 ProteoExtract subcellular fractionation kit (Merk Millipore) following the manufacturer's
493 instructions. Total cell protein and membrane fractions were separated by polyacrylamide
494 gel electrophoresis (PAGE) on 4-10% denaturing and non-denaturing TGX stain-free gels
495 (Bio-Rad) and transferred onto Immun-Blot PVDF membranes (Bio-Rad). The membranes

496 were blocked with 5% (w/v) dry milk-PBS overnight, rinsed with PBS and probed with
497 Adv5 (1×10^8 vp in 10 ml of milk-PBS) for 90 minutes. The membranes were then washed
498 three times with PBS and incubated with 10 μ g of biotinylated goat anti-Adv5 (Serotec) in
499 10 ml of milk-PBS for 60 minutes. The membranes were washed three times and
500 incubated with 10 μ g of streptavidin-conjugated horse radish peroxidase (HRP, Dako) in
501 10 ml of milk-PBS for 60 minutes. After extensive washing, the membranes were
502 developed with Immun-Star WesternC substrate (Bio-Rad) and visualized using a
503 ChemiDocMP digital imager (Bio-Rad). For competition VOPBA and before the addition of
504 Adv5, 10 μ g of rabbit anti-CAR pAb (Abcam) was added to the membranes and incubated
505 for 90 min at room temperature. After washing three times with PBS, the membranes were
506 probed with Adv5 and the assay carried out as described above.

507 For detection of CAR by Western blot, membrane fractions separated by PAGE were
508 transferred onto PVDF membranes, blocked with milk-PBS and probed with 10 μ g of rabbit
509 anti-CAR pAb or 1 μ g of anti- β actin mouse mAb (Abcam) in 10 ml of milk-PBS. After
510 washing with PBS containing 1% Tween 20 (PBS-T), the membranes were incubated with
511 1 μ g of anti-goat or anti-mouse antibody conjugated to HRP (Dako) in 10 ml of milk-PBS
512 for 60 minutes. After extensive washing with PBS-T, the membranes were developed as
513 described above.

514 Biochemical inhibitors.

515 The following inhibitors and final concentrations were used to block endocytosis:
516 cytochalasin D (CCD, 1 μ M, actin-dependent (Sakr *et al.*, 2001)); filipin (5 μ g/ml caveolae-
517 dependent (Rothberg *et al.*, 1992)); chlorpromazine (CPZ, 10 μ g/ml, prevents clathrin-
518 coated pit formation (Wang *et al.*, 1993)); methyl- β -cyclodextrin (m β -CD, 10 mM,
519 cholesterol-dependent (Vieth *et al.*, 2010)); amiloride (1 mM, Na⁺ blocker (West *et al.*,

1989), and therefore blocks macropinocytosis (Sallusto *et al.*, 1995)); ciliobrevin (10 μ M, inhibitor of motor cytoplasmatic dynein (Firestone *et al.*, 2012); dynasore (8 mM, inhibitor of dynamin and clathrin-dependent endocytosis (Macia *et al.*, 2006)).

The following lysosomotropic agents were used to block acidification of endosomal compartments: bafilomycin (1 μ M, inhibitor of vacuolar-type H⁺-ATPase (Yoshimori *et al.*, 1991); NH₄Cl (a weak base (Sonawane *et al.*, 2002) and diluted in PBS); chloroquine (10 μ M, inhibits endosomal maturation (Mellman *et al.*, 1986)).

All inhibitors were diluted in dimethyl sulfoxide (DMSO), except where noted. DMSO was used as diluent control and PBS was used as a negative control. All chemicals were obtained from Sigma-Aldrich (Poole, UK). Cells (1x10⁶ final) were plated in triplicate in culture media in 96 U-bottomed plates, the biochemical inhibitors added to the final concentrations described above in a final volume of 100 μ l and mixed thoroughly. After 1hr incubation at 37°C, virus internalization assays were performed as described below.

Virus internalization assay.

To differentiate between attachment and entry, a fluorometry phagocytosis assay (Wan *et al.*, 1993) based on the capacity of trypan blue to quench extracellular fluorescein was modified. AdV5 was labelled with NHS-fluorescein (Pierce) following the manufacturer's instructions. ALDC or 293 cells were cultured in 96-well plates with the labelled virus (moi=100 vp/cell) for 6 hrs or 60 min respectively at 37°C or at 4°C in the presence of the biochemical inhibitors described above. All inhibitors prepared in DMSO were diluted in culture media and 10% DMSO in culture media was used as negative control. After the required incubation period, trypan blue (0.5% final, Sigma) and Live/Dead Aqua (Invitrogen) were added and the cells analysed by flow cytometry; 25,000 live/single events were used to generate statistical analyses.

544 Confocal microscopy

545 FACS purified DC (FSC^{high} MHCII⁺ DEC-205⁺ CD11c⁺ CD8α⁻) were cultured on collagen-
546 treated coverslips (Sigma) in the presence (moi = 10) or absence of labelled AdV5. The
547 cells were fixed with 3% paraformaldehyde for 20 minutes, washed twice with PBS and
548 permeabilized with 0.1% Triton X-100 in PBS. Anti-EEA1 polyclonal antibody raised in
549 rabbits (1 µg/ml final concentration, Abcam, UK) was used to visualize early endosomal
550 compartments; the tracer molecules dextran and albumin (both at 0.5 µg/ml final
551 concentration) conjugated to AF488 were used for co-localization assays; rabbit anti-
552 FMDV was used as a negative control (final dilution of 1:750). Goat anti-rabbit-AF488 (1
553 µg/ml, Invitrogen) was used as secondary antibody and all samples were counterstained
554 with DAPI (100 nM, Invitrogen) following the manufacturer's instructions. Where indicated,
555 Phalloidin-AF488 (0.2 U/slide, Invitrogen) was used to identify actin filaments. Cells were
556 mounted onto microscope slides using VectaShield (Vector Laboratories, UK) and
557 observed using a 65x lens mounted on a Leica SP5 confocal microscope. The Leica LAS
558 AF software was used to take sequential, 3D stack images in the Z-plane acquiring stacks
559 of 80 to 120 optical sections from infected cells as optimized by the software. The number
560 of intracellular virions, 3D images and co-localization datasets were analysed using
561 Bitplane Imaris 6.4.2 image analysis software (Bitplane, Switzerland) with surface
562 smoothing of 0.05 nm for nuclei (blue), 0.02 nm for AdV (red) and endosomes (green).

563 pH-dependent fluorometry

564 Dextran-fluorescein (Invitrogen) was used to measure intracellular pH essentially as
565 described previously (Downey *et al.*, 1999). Calibration of the fluorescence ratio versus pH
566 was performed for each experiment by equilibrating the cells in isotonic K⁺-rich medium
567 buffered to varying pH values (between 5.0 and 7.5) in the presence of the K⁺/H⁺
568 ionophore nigericin (5 mM, Sigma). Calibration curves were constructed by plotting the

extracellular pH, which was assumed to be identical to the cytosolic pH under these conditions, against the corresponding fluorescence ratio. AdV5 (moi = 100) or dextran (25 µg/ml) labelled with fluorescein were added to ALDC or 293 cells cultured in triplicate in 96-well plates (Costar). Real time fluorometry was measured every 30 minutes using an Infinite M200 (Tecan) and the results analysed using Magellan for Windows (Tecan).

Generation of oil-in-water emulsions

AdV5 recombinants expressing GFP or Ag85A were mixed with the adjuvant Montanide ISA 206V (SEPPIC, France) to form oil-in-water emulsions following the manufacturer's instructions. Briefly, 1×10^9 vp in a volume of 250 µl were mixed with an equal volume of adjuvant and vigorously mixed for 2 minutes. Two negative controls were also prepared, one containing AdV5 and adjuvant but without mixing, and the other PBS mixed with ISA 206V and emulsified as described above. The emulsions containing 100 vp/cell were added to 96-well U-bottomed tissue culture plates containing 1×10^5 ALDC in 100 µl of media and mixed by pipetting until the solution looked homogenous. In the case of the control containing AdV5 and adjuvant without mixing, the solutions were mixed only once and clear hydrophobic/hydrophilic globules observed macroscopically. The infection allowed to continue as described in the text and washed twice with PBS before analysis.

Antigen presentation assays

Ag85A-specific IFN-γ producing lymphocytes were analysed using ELISpot assays as described previously (Guzman *et al.*, 2012; Hope *et al.*, 2012) utilizing purified CD4⁺ T cells from MHC-matched, BCG-vaccinated animals (Thom *et al.*, 2012).

Statistical analysis

Calculation of descriptive statistics (geometric statistics, standard error of the means and standard deviations), two-way parametric analysis of variants (ANOVA) including multiple

593 comparisons, Bonferroni multiple comparison tests and graphs were generated using
594 GraphPad Prism for Windows v6.01 (GraphPad, San Diego, CA).

595

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602

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844

845

846 **FIGURE LEGENDS**

847 **Figure 1.** Phenotype of ALDC and expression of CAR in bovine cells. a) The phenotype of
848 ALDC was characterized as FSC^{high} MHC class II⁺ DEC205⁺ CD11c⁺ CD8α⁻ and 91% of
849 these cells were CD172a⁺. The plot is representative of all samples analysed (n = 8
850 animals). b) Expression of CAR and β-actin was assessed by Western blot on enriched
851 membrane fractions from 293, bovine lung (BL) or ALDC. Blot representative of 3 different
852 experiments. c and d) Virus overlay protein binding assay (VOPBA) was used to confirm
853 binding of AdV5 to enriched membrane fractions from 293 and BL but not from ALDC
854 under denaturing and non-denaturing conditions. e) Competition VOPBA using anti-CAR
855 antibodies to block binding of AdV5 to enriched membrane fractions from 293 and BL
856 cells. Blots representative of 5 different experiments.

857

858 **Figure 2.** Transduction of ALDC by AdV5-GFP following removal of putative receptors
859 from the cells' surface. a-f) Expression of putative AdV5 receptors on ALDC 24 hrs after
860 treatment (doted histograms) or mock treatment (solid histograms); grey-filled histograms:
861 isotype controls. g-l) Expression of GFP in AdV5-GFP transduced untreated (solid
862 histograms), treated (doted histograms) or mock transduced ALDC (grey-filled
863 histograms). m and n) Expression of GFP in AdV5-GFP-transduced ALDC and 293 cells in
864 the presence of the RGD antagonist Cyclo(Ala-Arg-Gly-Asp-3-Aminomethylbenzoyl (doted
865 histogram) or control peptide (solid histogram); grey-filled histogram: mock transduced
866 cells. o and p) Expression of GFP in AdV5-GFP-transduced ALDC and 293 in the
867 presence of AdV5-hyperimmune bovine sera (doted histograms), normal bovine serum
868 (solid histograms) or mock transduced (grey-filled histograms). Plots are representative of
869 cells from 6 different animals analysed in duplicate.

870 **Figure 3.** AdV5 entry and trans-gene expression in ALDC is significantly different from that
871 in CAR-expressing 293 cells. ALDC (white circles) and 293 cells (black squares) were
872 cultured on ice (4°C) or at 37°C with biotinylated AdV5-GFP for 90 minutes followed by
873 washing off the inoculum. a) Biotinylated membrane-bound virus was detected by ELISA
874 using streptavidin-HRP. b) Cells were transduced as before and cultured for 24 hrs at
875 37°C; expression of the trans-gene (GFP) was detected by flow cytometry. c) ALDC and
876 293 cells were cultured at 37°C in the presence of AdV5-GFP. At 1 hr intervals, aliquots
877 were washed with PBS and the cells allowed to recover overnight after which GFP
878 expression was measured by flow cytometry. d) ALDC and 293 cells were cultured at 37°C
879 in the presence of AdV5-GFP and without washing; GFP expression was measured by
880 flow cytometry at 1 hr intervals. Each point represents the mean of cells from 6 different
881 animals tested in duplicate and error bars indicate standard error of the mean.

882 **Figure 4.** Uptake of AdV in DC is blocked by inhibitors of actin-dependent endocytosis.
883 ALDC and 293 cells were cultured with AdV5-Fluo for 1 hr at 37°C in the presence of
884 various endocytosis inhibitors as described in materials and methods. Extracellular
885 fluorescein was then quenched with trypan blue and fluorescence was measured by flow
886 cytometry. A) Representative histograms showing fluorescence after various treatments.
887 The markers indicate percentage of fluorescein-positive cells above background. b) Bar
888 graphs showing means and standard deviations of the percentage of fluorescein⁺ ALDC
889 obtained from 6 different animals and tested in duplicate or 293 cells tested in triplicate.
890 Asterisks indicate *=p<0.05 and **=p<0.005 compared to treatment with DMSO.

891 **Figure 5.** Entry of AdV5 into ALDC. ALDC were cultured at 37°C in the presence of
892 AF568-labelled AdV5 (moi = 100). Cells were fixed and analysed by confocal microscopy
893 at 3 hrs (a) 4hr (b) or 5 hrs (d) post infection. Blue: DAPI; Green: Phalloidin-AF488 (for F-

894 actin); Red: AdV5-AF568. Representative samples of cells from 5 different animals. The
895 scale bar represents 20 μ m.

896

897 **Figure 6.** The majority of AdV are not associated with early endosomes. ALDC were
898 cultured with AdV5-AF568 (red) and Dextran-AF488 (green) or albumin-AF488 (green) for
899 4 hrs and fixed. The early endosome marker (EEA1) was detected using monospecific
900 antibodies and AF488 secondary antibodies (green) as described in Materials and
901 Methods and the samples analysed by confocal microscopy. a) Colocalization of AdV5 and
902 EEA1; b) AdV5 and dextran; d) AdV5 and albumin. Blue: DAPI; Yellow: co-localization.
903 Inserts show a higher magnification of the region of interest. Arrows indicate colocalization
904 events. Representative samples cells from 4 different animals. d) Bar graph showing mean
905 percentage of co-localization voxels from single-slice histograms across the Z-plane from
906 at least 10 confocal images processed from cells from 4 different animals. Error bars
907 indicate standard error of the mean.

908 **Figure 7.** Acidification of endosomes is not required for transduction of ALDC by AdV5. a)
909 ALDC or 293 cells were incubated with AdV5-GFP for 24 hrs in the presence of various
910 inhibitors of intracellular acidic microenvironment or with the diluents DMSO or PBS.
911 Trans-gene expression (GFP) was measured by flow cytometry. b) ALDC were cultured at
912 37°C with fluorescein-labelled AdV5 or dextran for 4 hrs in the presence of normal tissue
913 culture media or media containing 10 mM NH_4Cl pH 7.5. After washing fluorescein mean
914 fluorescence intensity (MFI) was measured by flow cytometry. c) Relative fluorescence
915 standard curve of fluorescein-dextran at various pH. D) ALDC were cultured in the
916 presence of fluorescein-labelled AdV5 or dextran. Relative fluorescence (RFU) was
917 measured by real time fluorometry. Each point represents the mean of cells from 4
918 different animals tested in duplicate and error bars indicate standard error of the mean.

919 **Figure 8.** Oil-in-water adjuvants improve AdV5 uptake by ALDC, increase and prolong
920 recombinant antigen expression and enhance antigen presentation. AF568-labelled AdV5
921 was mixed with PBS or an oil-in-water adjuvant, added to ALDC, cultured overnight and
922 washed twice prior to analysis. a) Confocal microscopy was used to count the number of
923 intracellular virions/cell 4 hrs post infection. b and c) 3D reconstructions of representative
924 samples of ALDC infected with AdV5-568 in PBS or adjuvant respectively 4 hrs post
925 infection. d) GFP mean fluorescence intensity (MFI) of ALDC infected with AdV5-GFP in
926 PBS or adjuvant 24 hrs post infection. e) Frequency of GFP-expressing ALDC over time
927 following infection with AdV5-GFP in PBS or adjuvant. f) Ag85A-specific IFN- γ ELISpot
928 using AdV5-Ag85A or AdV5-GFP in PBS or adjuvant. ALDC were infected with AdV5
929 recombinants as described in Materials and Methods and cultured with MHC-matched
930 CD4⁺ T cells from BCG-vaccinated animals. In all cases the bars indicate means of 20
931 imaged cells from 3 different animals analysed in duplicate and error bars indicate
932 standard error of the means.

Figure 1

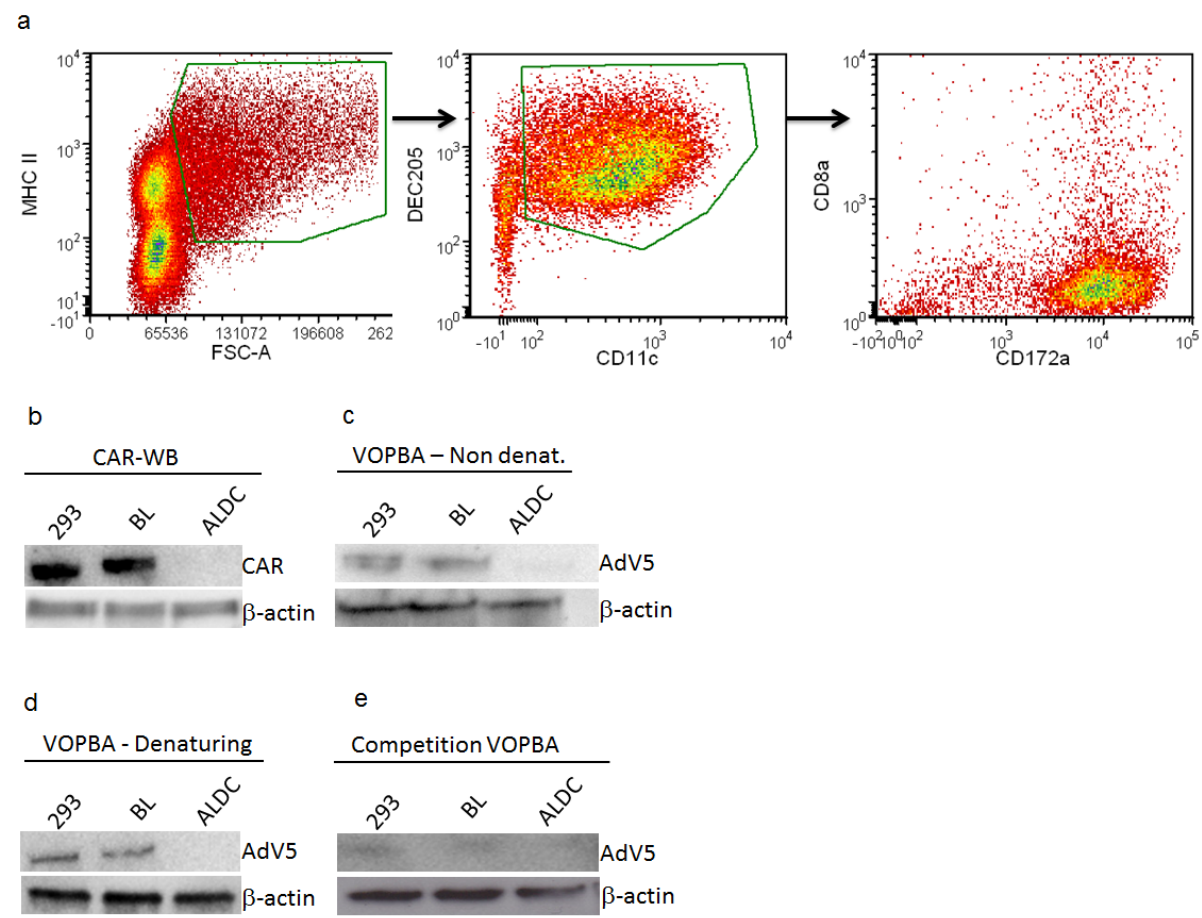


Figure 2

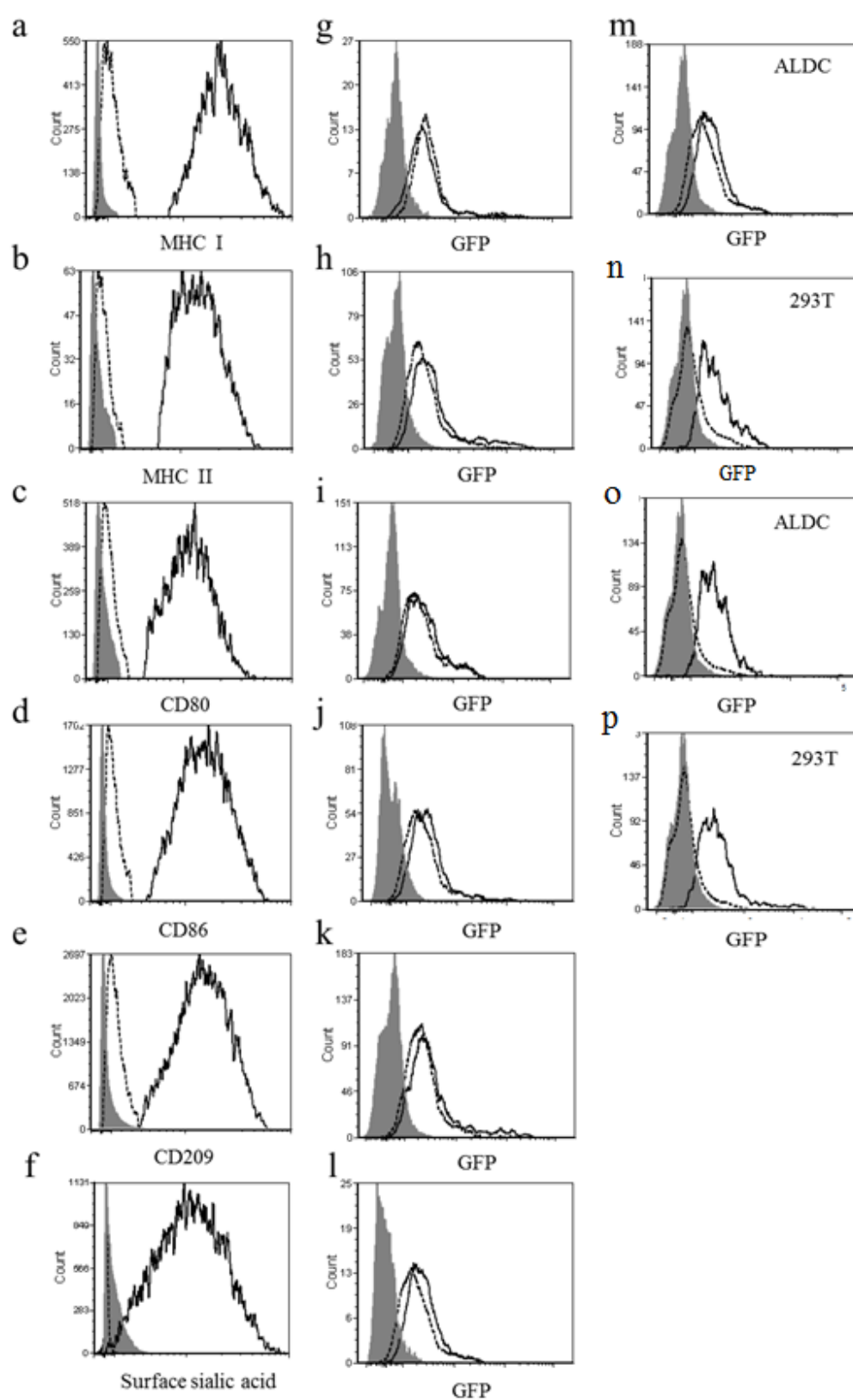


Figure 3

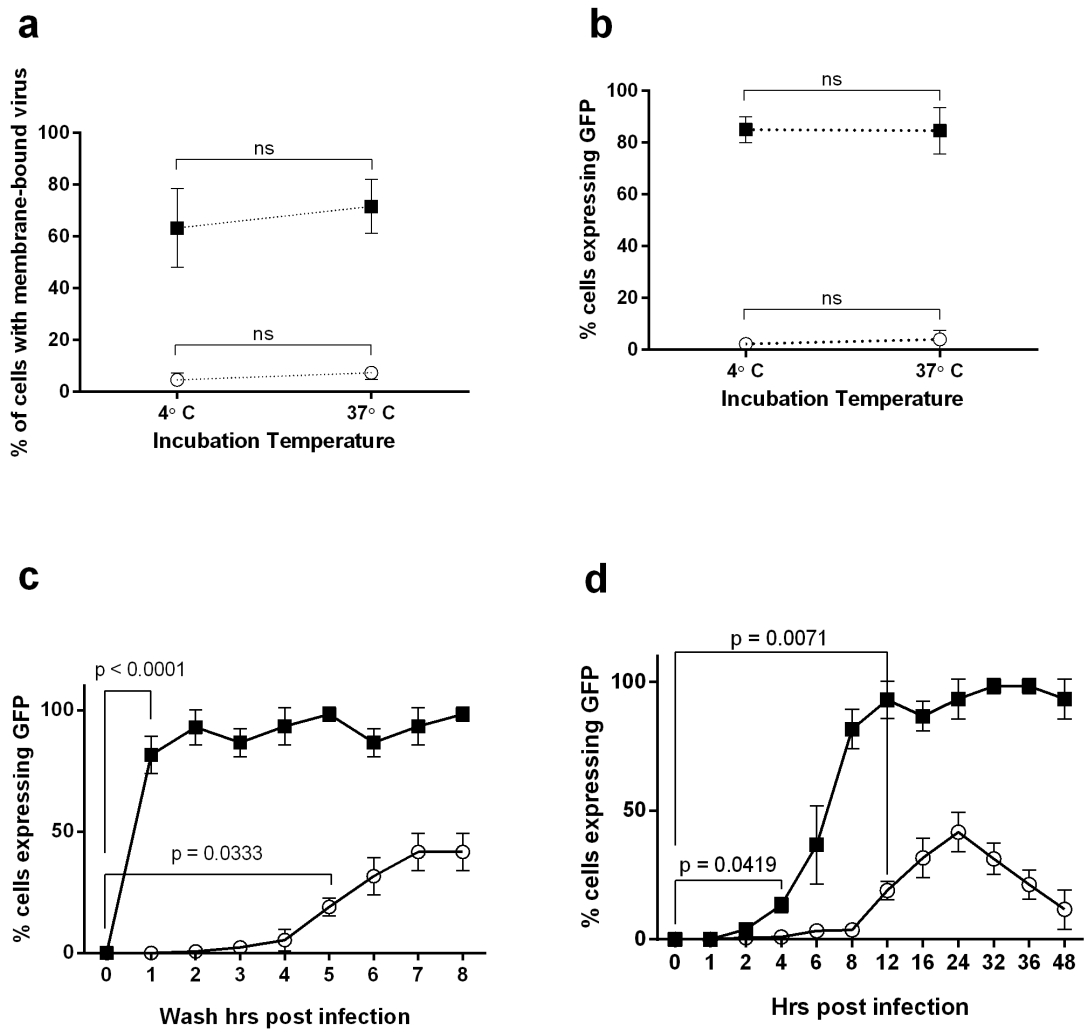
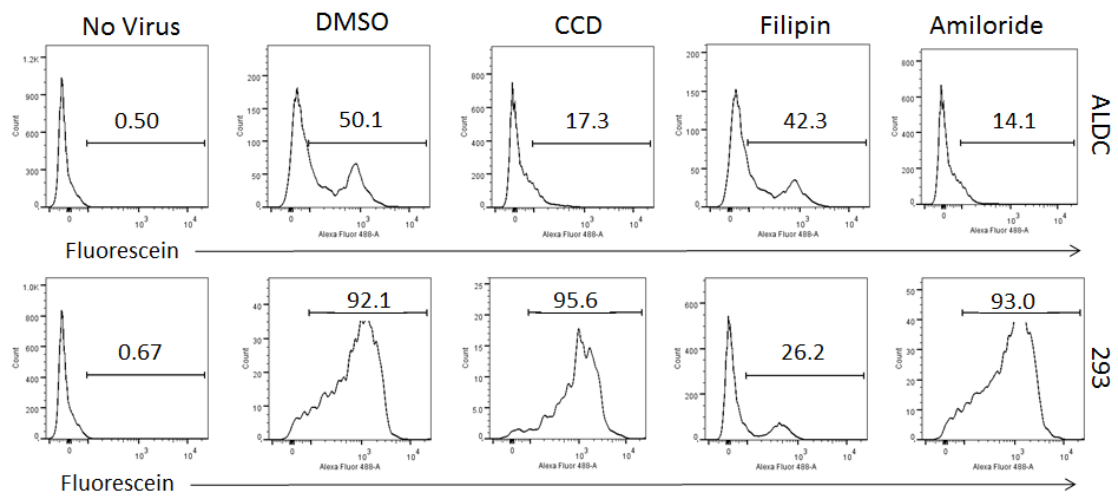


Figure 4

a



b

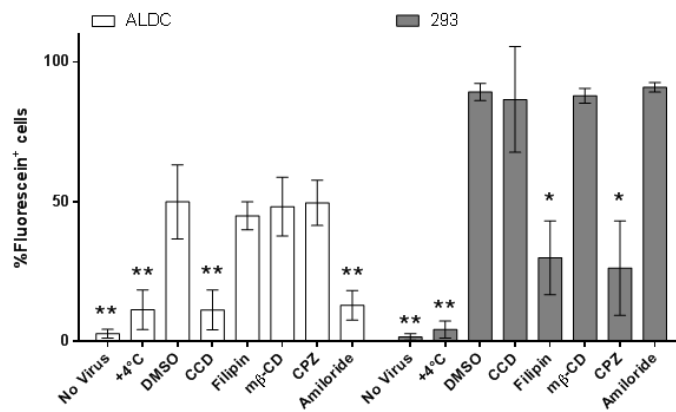


Figure 5

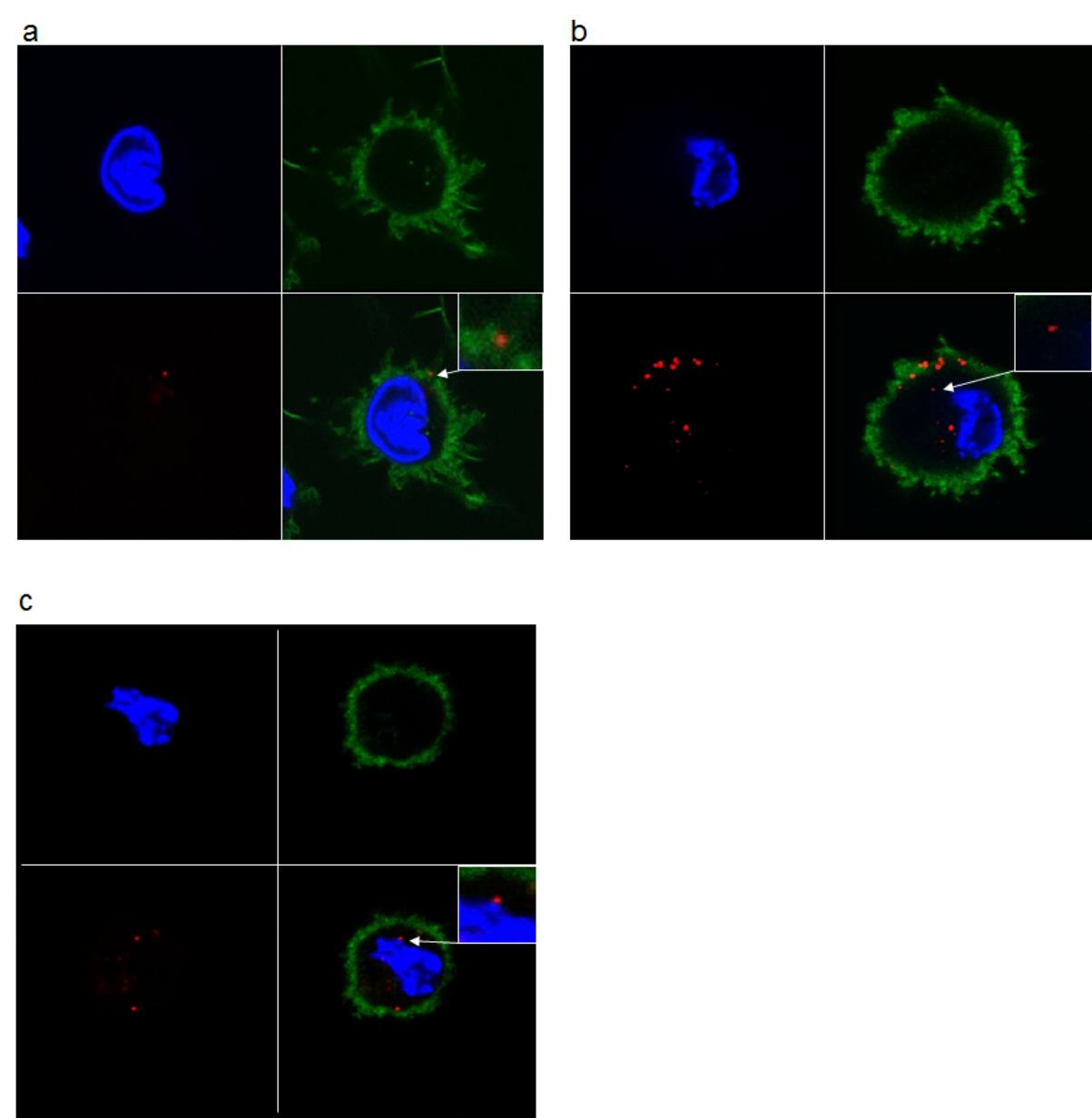


Figure 6

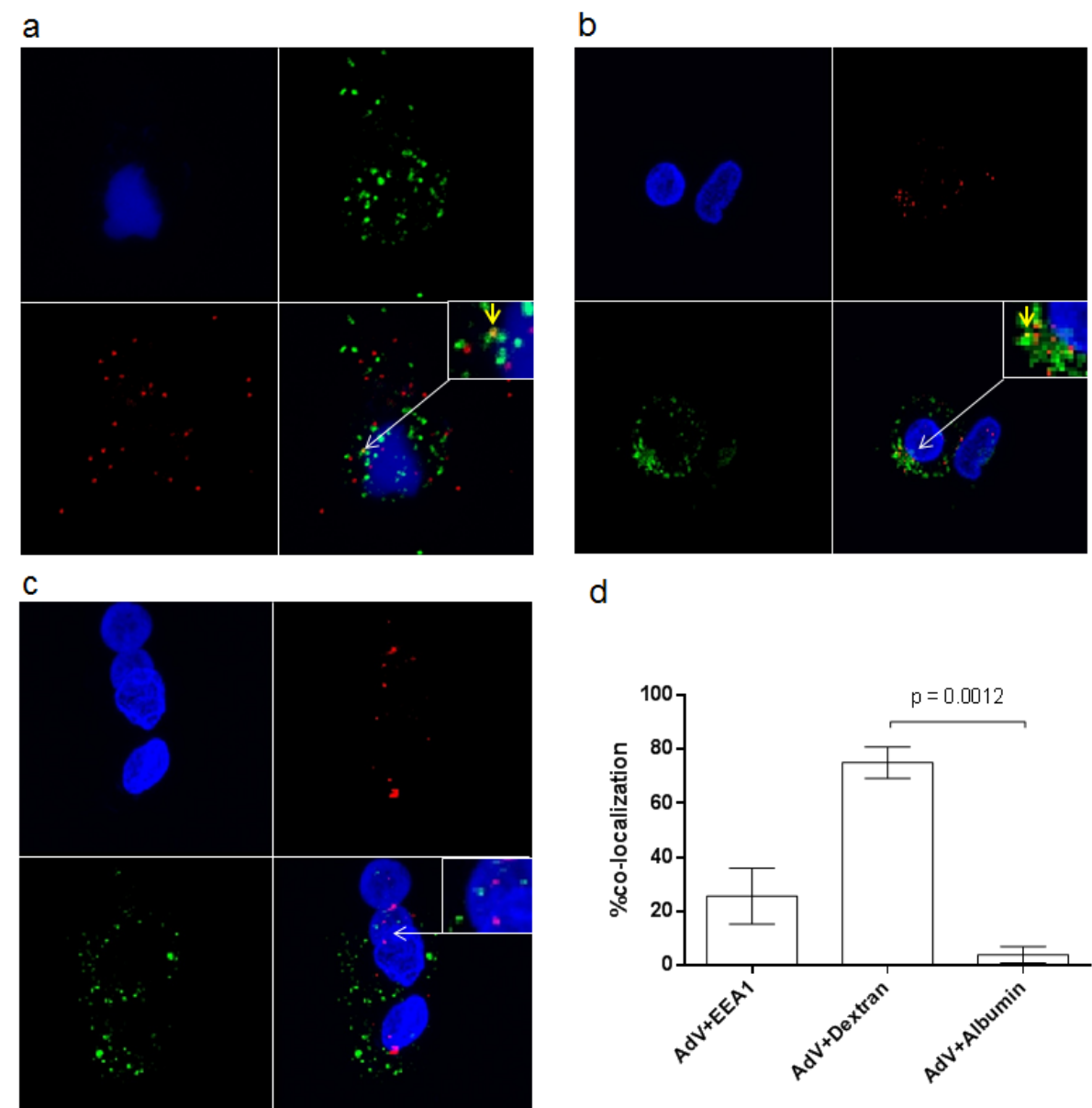


Figure 7

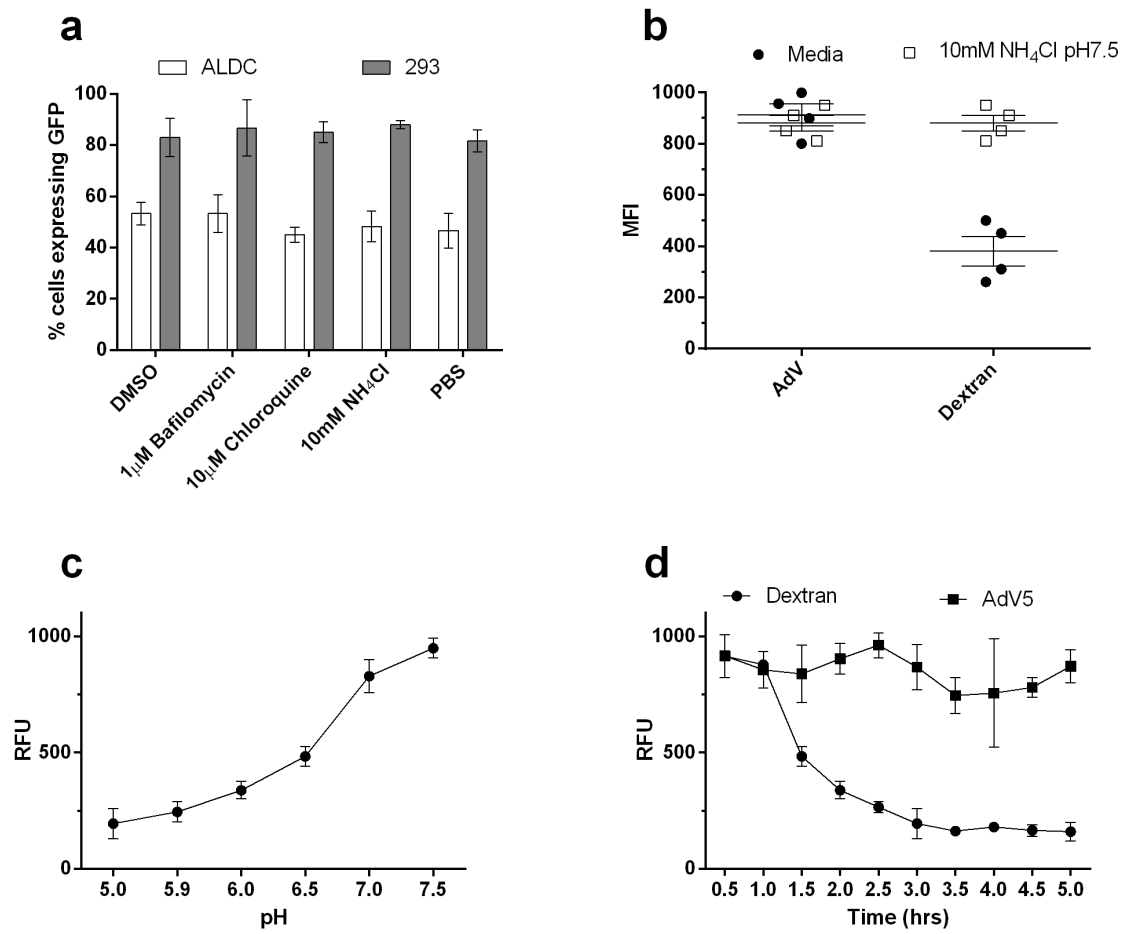
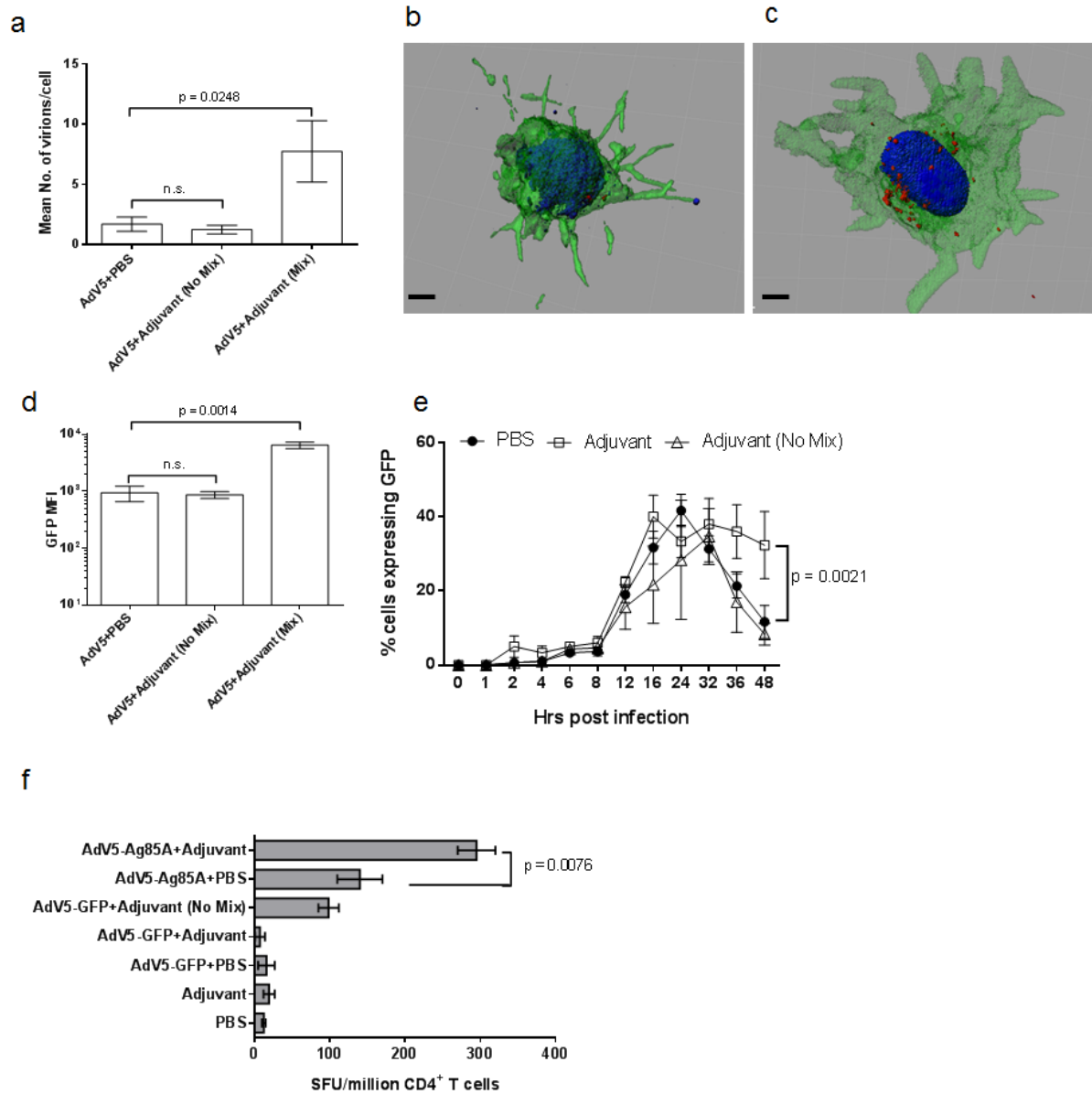
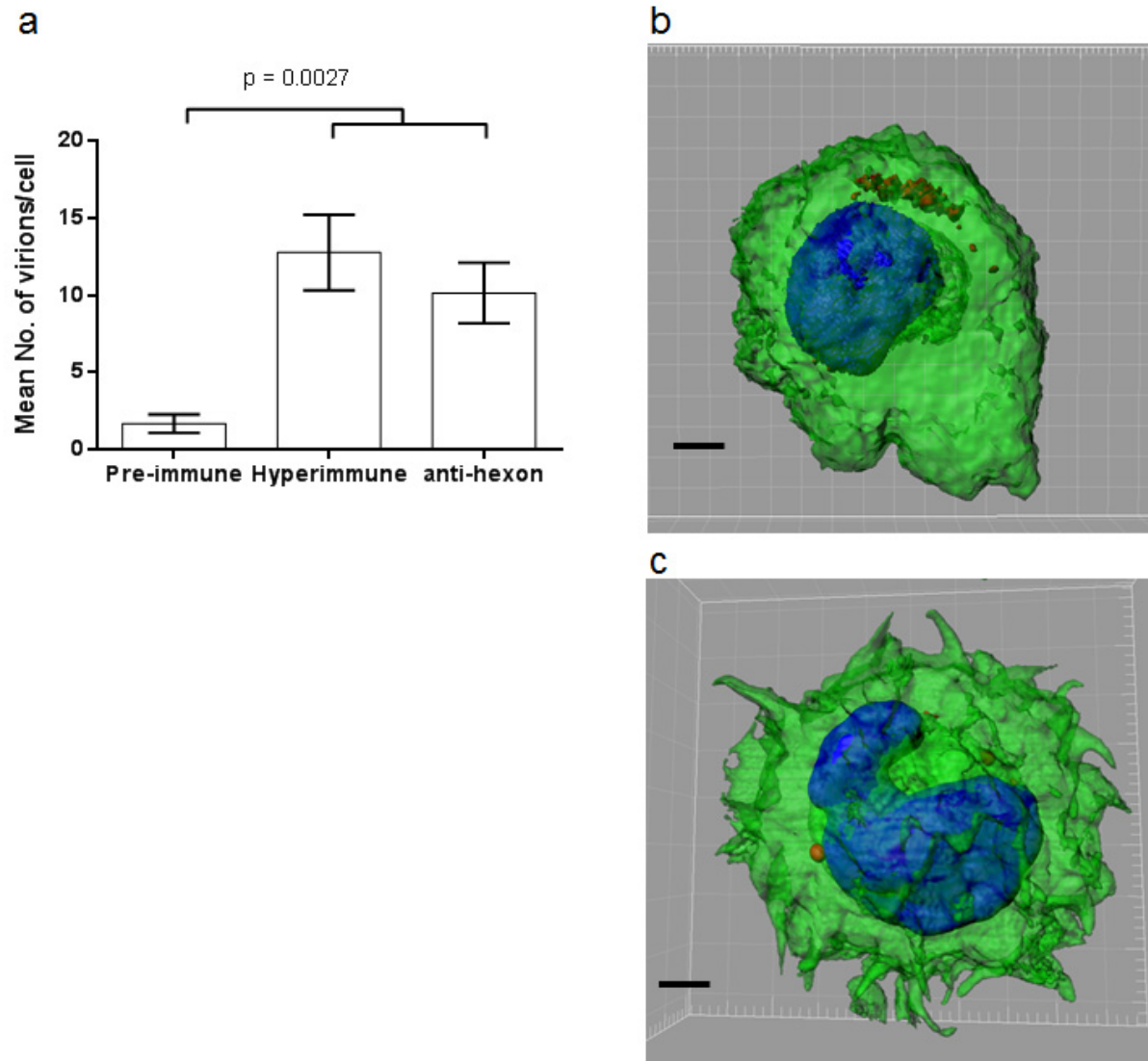


Figure 8



Supplementary Figure 1



S1. Neutralizing antibodies do not prevent virus entry into ALDC. AF568-labelled AdV5 was mixed with PBS, normal bovine sera or bovine hyperimmune sera to AdV5 and incubated for 60 min after which the mix was added to ALDC as described in Materials and Methods and confocal microscopy was used to quantify the number of intracellular AF568-labelled virions. a) Mean number of intracellular AF568⁺ particles per cell. Bars indicate means of cells from 5 different animals analysed in duplicate and error bars indicate standard error of the means. b and d) Three-dimensional reconstructions from confocal micrographs showing ALDC cultured for 5 hrs with AdV5-AF568 previously incubated with hyperimmune sera (b) or normal sera (c). Reconstructions are representative of cells from 5 independent experiments. Black bar indicates 5 microns.